Autoregulation of Nisin Biosynthesis in Lactococcus lactis by Signal Transduction*

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The post-translationally modified, antimicrobial peptide nisin is secreted by strains of Lactococcus lactis that contain the chromosomally located nisin biosynthetic gene cluster nisABTCIPRKFEG. When a 4-base pair deletion is introduced into the structural nisA gene (ΔnisA), transcription of ΔnisA is abolished. Transcription of the ΔnisA gene is restored by adding subinhibitory amounts of nisin, nisin mutants, or nisin analogs to the culture medium, but not by the unmodified precursor peptide or by several other antimicrobial peptides. Upon disruption of the nisK gene, which encodes a putative sensor protein that belongs to the class of two-component regulators, transcription of ΔnisA was no longer inducible by nisin. Fusion of a nisA promoter fragment to the promoterless reporter gene gusA resulted in expression of gusA in L. lactis NZ9800 (ΔnisA) only upon induction with nisin species. The expression level of gusA was directly related to the amount of inducer that was added extracellularly. These results provide insight into a new mechanism of autoregulation through signal transduction in prokaryotes and demonstrate that antimicrobial peptides can exert a second function as signaling molecules.

Nisin is an antimicrobial peptide (1–3) widely used in the food industry as a safe and natural preservative. The ribosomally synthesized precursor peptide undergoes extensive post-translational modification, which includes dehydration of serine and threonine residues and the formation of thioether bridges called (β-methyl)lanthionines, resulting in five ring structures named A, B, C, D, and E (Fig. 1B). Peptides containing these characteristic modified residues are named lantibiotics (4). Eleven genes organized in a cluster have been implicated to be involved in the complex biosynthesis of nisin, i.e. nisABTCIPRKFEG (Fig. 1A) (5–11). Of these genes, nisE encodes the nisin A precursor peptide of 57 amino acid residues; nisB and nisC encode putative enzymes involved in the post-translational modification reactions (based on homology to genes found exclusively in other lantibiotic gene clusters); nisT encodes a putative transport protein of the ABC translocator family that is probably involved in the extrusion of modified precursor nisin (7, 9); nisP encodes an extracellular subtilisin-like protease involved in precursor processing (8); nisI encodes a lipoprotein involved in the producer self-protection against nisin (9); and nisFEG encodes putative transporter proteins that have also been implied in immunity (11). A schematic representation of the post-translational events yielding mature nisin A is shown in Fig. 1B. Nisin Z is a natural variant of nisin A that contains an asparagine residue at position 27 instead of the histidine residue found in nisin A (12). Both nisin A- and nisin Z-producing strains are common in nature, and both structural genes (nisA and nisZ) have been cloned (5, 6, 13).

The proteins encoded by nisR (8) and nisK (10) have shown to be involved in the regulation of nisin biosynthesis (8, 10). NisR is a response regulator, and NisK is a sensor histidine kinase which belong to the class of two-component regulatory systems (14–16). When the genes nisABTCIR are present on a multicopy plasmid, production of fully modified precursor nisin is observed, indicating that overexpression of nisR alone is sufficient to activate transcription of nisA and obviously also of the biosynthetic genes downstream by partially reading through an inverted repeat sequence (Fig. 1A) (8). This observation is similar to the regulation of expression of iep and degU genes in Bacillus subtilis, where overexpression of the response regulator activates transcription of the target genes (17), and to the case of overexpression of epiQ, which encodes a response regulator involved in the biosynthesis of the lantibiotic epidermin (18). When only the genes nisABTCIR are present on a multicopy plasmid (pNZ9000) in Lactococcus lactis MG1614, no transcription of nisA is observed (9). Two gene products have been identified for the regulation of the biosynthesis of the related lantibiotic subtilin (19), which also belong to the class of two-component regulators, i.e. SpaR, the response regulator, and SpaK, the sensor histidine kinase (20, 21). Upon disruption of either of these genes, subtilin production was abolished, indicating the involvement of these gene products in subtilin biosynthesis (20). The regulation was shown to be growth-phase dependent, but an inducing signal was not identified (20, 21).

While the structure and function of two-component regulators have been studied in great detail (14–16), the nature of the inducing signal has remained unclear in many cases. It is demonstrated here that fully modified nisin can induce the transcription of its own structural gene as well as of the downstream genes by limited read-through, via signal transduction, by acting as the extracellular signal for the sensor histidine kinase NisK.

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MATERIALS AND METHODS

Strains and Plasmids—L. lactis strains MG1614 (22), NZ9700 (a nisin-producing transconjugant containing Tn5276) (23), and NZ9800

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FIG. 1. A, organization of the nisin gene cluster. Established (nisAlPRKFEG) and putative (nisBCT) functions of the gene products have been indicated. P denotes a mapped promoter, and IR denotes an extensive inverted repeat sequence that could act as a rho-independent terminator (7).

B, schematic outline of the biosynthesis of nisin A. Rings are labeled A–E. Asterisks indicate residues that will be modified. The black arrow indicates processing of the N-terminal Met residue, while the small white arrow indicates processing of the leader peptide by the action of NisP (8). Dha, dehydroalanine; Dhb, dehydrobutyrylne.
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(a derivative of N29700 in which the nisA gene has been exchanged by replacement recombination with a modified nisA gene containing a 4-bp 1 deletion in the proincon-encoding part (ΔnisA) and which is therefore no longer able to produce nisin A) have been described previously (9). L. lactis strains were cultivated without aeration at 30 °C in M17 broth (Difco) supplemented with 0.5% (w/v) glucose (GM17) or sucrose (GM17s). DNA, lysis of erythromycin-resistant colonies, and plasmids (24), media were supplemented with 10 μg/ml chloramphenicol. Expression plasmids pNZ9010 and pNZ9013 (9, 25), containing the nisA and nisZ genes, respectively, under control of the efficient lac promoter, were introduced into L. lactis strain N29800, leading to the production of nisin A or nisin Z in similar amounts as in L. lactis with pNZ9012. For this host strain for cloning experiments, E. coli strain MC1061 (26) was used.

The nisA promoter region including part of the nisA gene was isolated as a 1.442-bp BglII-EcoD136I fragment from plasmid pNZ9000 (8). This fragment was cloned into pNZ273, containing the promoterless gusA gene (24), which had been digested with BglII and Scal, generating plasmid pNZ82003. Part of the upstream promoter region was deleted by digesting pNZ82003 with BglII and Thnl111. These sites were made blunt by Klenow polymerase and ligated, generating plasmid pNZ82008, which eventually contained a 312-bp nisA promoter fragment in front of the gusA gene. Another part of the nisA promoter region, including all the nisA gene and the first part of the nisB gene, was isolated as a 1.043-bp BglII-HinM1111 fragment from plasmid pNZ82008. This fragment was cloned into pNZ277 (24), which had been digested with BglII and EcoRI, generating plasmid pNZ82002. A 1.442-bp BglII-EcoD136I fragment was added to pNZ82002, generating pNZ82001, by making the BglII site blunt with Klenow polymerase and subsequent ligation to the EcoD136I site. All constructs were initially made in E. coli MC1061 (26). Plasmids pNZ82005, pNZ82002, and pNZ82001 were used to transform L. lactis NZ9700 and L. lactis NZ9800 (9), and transformants were obtained by selecting for resistance to chloramphenicol.

DNA Techniques and DNA Sequence Analysis—Restriction enzymes and other DNA-modifying enzymes were purchased from Life Technologies, Inc. or U. S. Biochemical Corp. and used as recommended by the manufacturers. The DNA sequence of the nis region was determined on the double-stranded plasmid DNA with the HindIII primer by the chain termination method (27). Transcription analyses of the nisA and ΔnisA genes were performed by isolation of RNA from L. lactis strains N29700 and N29800, Northern blotting, and subsequent hybridization with a radiolabeled nisA probe as described previously (9). RNA isolation was performed 2 h after induction of a culture with an A600 nm of 0.5. RNA (20 μg) was loaded in each lane, and the amounts were estimated by comparing the intensity of the 16 S and 23 S RNA bands.

Inactivation of Chromosomal nisK and nisG by Gene Replacement—The chromosomal copy of the nisK gene was inactivated by introduction of an erythromycin resistance gene (28) into the open reading frame of nisK. This was achieved by cloning a 2.8-kilobase pair HindIII-EcoRI chromosomal DNA fragment from strain N29700 containing the 5' part of the nisK gene and the intact nisR and nisK genes was cloned into pUC19. The erythromycin resistance gene was introduced into a unique SalI site, resulting in the interruption of the nisK open reading frame between the encoded amino acids 9 and 10 of nisK and leaving 1.1 and 1.7 kilobase pairs of flanking regions at the 5' and 3'-ends, respectively. This construct was designated pNZ29195. Strain N29800 was transformed with the nonreplicating plasmid pNZ29150, and integrants were selected on plates containing erythromycin (2.5 μg/ml). The selected integrants were analyzed by polymerase chain reaction using primers 5'-CGGCTAATCCGAGG-3' and 5'-CCGTTTGAATACTTCTCC-3' and by Southern hybridization using pUC19 DNA (29) as a probe. In one integrant (pNZ29800), the erythromycin resistance gene had been integrated via gene replacement at the 3' and 5'-flanking regions, introducing this gene into the open reading frame of the chromosomal copy of the nisK gene, in the absence of any pUC19 sequences. The resulting construct was further analyzed by polymerase chain reaction of the nisK region and by Southern blotting using nisK as a probe to confirm the correct integration configuration.

The nisB gene was disrupted by introducing a 162-bp in-frame deletion into the middle of the gene. This was accomplished by cloning a 4.4-kilobase pair BglII-EcoRI fragment, containing nisB and surrounding regions from the nisB gene cluster, into a BamHI-EcoRI-digested pUC19 vector, which harbored an additional erythromycin resistance marker, as has been described previously (9). The deletion was made by removing an internal HpaI fragment from the nisB gene and subsequent ligation. The resulting plasmid was named pNZ9135 and was used for transformation of L. lactis N29700. Following transformation, erythromycin-resistant colonies were obtained that had integrated the plasmid by recombination of the plasmid with one of the flanking regions of the deleted fragment. After growing for 200 generations in the absence of erythromycin, the plasmid pNZ273 was isolated from several clones. Plasmid pNZ273 was sensitive to erythromycin. This had apparently been caused by a second recombination event involving the flanking region on the other side of the deletion than the side of the first recombination event, resulting in the replacement of nisB with ΔnisB on the chromosome. The configuration of the desired construct was confirmed by polymerase chain reaction analysis of the nisB region with the deletion and by Southern analysis of BglII-digested chromosomal DNA. The desired strain was called N29700 ΔnisB.

Production, Purification, and Characterization of Nisin Mutants—Mutants of nisin Z were produced as described previously (25). The primers used for site-directed mutagenesis of the nisZ gene were as follows: 5'-CACCGTGCATACCGCTGGAACGATTTTCCGCTATGTC-3' (1W), 5'-CACCGACCTTACAGATTTCCGCTATGTC-3' (T2S), 5'-CACACCGATTACAAATTCTGCTATGCACACCC-3' (S3T), and 5'-AACAGGAGCTCTGGGTTGAACATC-3' (M1W) (mutated nucleotides are indicated in boldface). All mutants were purified to homogeneity, and the structures of the modified residues were confirmed by one- and two-dimensional 1H NMR (25). It was determined that T2S nisin Z contains a dehydroalanine residue at position 2, S3T nisin Z has a β-methylalanyline ring between residues 3 and 7 instead of a lanthionine, and 11W nisin Z and M17W nisin Z contain a Trp residue at positions 1 and 17, respectively. Precursor nisin Z, containing the subtilin leader peptide (sl-nisin Z), was obtained as described before (30). Purified lacticin 481 was isolated as described previously (31, 32). Purified PepS (33) was obtained from Dr. H.-G. Sahl (Bonn, Germany). Unmodified precor sor nisin A was obtained from the laboratory of Dr. G. Jung (Tübingen, Germany). Preparations of subtilin (19) and lactococcin A (34) consisted of culture supernatants of producing strains, which were confirmed to possess substantial antimicrobial activity in agar diffusion assays. Antimicrobial activities against the selected strains were determined as described before for Micrococcus flavus (25). L. lactis was cultured in GM17 broth at 30 °C with an initial A600 nm of 0.025, and outgrowth was measured when the culture without nisin had reached an A600 nm of 0.8.

β-Glucuronidase Assays—Lactococcal cells (1 ml) were harvested at 1.5 h after induction with nisin (or mutants or fragments) or other antimicrobial species and adjusted to 0.1 μl of a 0.025, 0.8 mM EDTA, and 10 μM β-nitro-β-D-glucu ronic acid (Clontech, Palo Alto, CA). The mixture was incubated, and the increase in A405 nm was measured at 37 °C. Histochromical screening for gusA positive colonies was performed by including 5-bromo-4-chloro-3-indolyl glucuronide (Research Organics Inc., Cleveland, OH) at a final concentration of 0.5 mg/ml in GM17 plates (24). Purified nisin fragments (35–39) were obtained from Prof. T. Shibata (Protein Research Foundation, Osaka, Japan).

RESULTS AND DISCUSSION

Transcription Analyses of nisA in the Presence and Absence of Nisin or Nisin Mutants—The promoter sequence and the transcription start site of nisA have been identified, and a transcription unit of ~260 nucleotides has been demonstrated in L. lactis strain N29700, which contains Tn5276 (9). It has also been found that transcription of nisA is dependent on the integrity of nisA itself since a 4-bp deletion in the middle of the nisA gene (ΔnisA) on the chromosome of L. lactis strain N29800 completely abolishes transcription of this gene (9). For further transcription analyses of the structural and biosynthetic genes, a series of isogenic lactococcal strains was used, including the nisin-producing N29700 and non-nisin-producing N29800 strains.

Northern blotting showed that in strain N29800, the transcript of nisA was absent, but after adding small amounts of

1 The abbreviation used is: bp, base pair(s).
against

ship between antimicrobial activity of the nisin mutants
lin,lacticin481,andlactococcinA).Thereisnodirectrelation-

(observation that antimicrobial activity is dependent on pore-

The difference in potency can be attributed to the
observation that antimicrobial activity is dependent on pore-

Table I. Comparison of induction capacity with antimicrobial activity against
L. lactis strain MG1614 or against M. flavus of nisin Z, nisin Z
mutants, and synthetic nisin A fragments (36)

<table>
<thead>
<tr>
<th>Nisin Z (mutant)</th>
<th>Induction capacity a</th>
<th>Activity against L. lactis b</th>
<th>Rings present</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2S nisin Z</td>
<td>1100</td>
<td>78</td>
<td>All</td>
</tr>
<tr>
<td>M17W nisin Z</td>
<td>220</td>
<td>12</td>
<td>All</td>
</tr>
<tr>
<td>Nisin Z</td>
<td>100</td>
<td>100</td>
<td>All</td>
</tr>
<tr>
<td>S3T nisin Z</td>
<td>11</td>
<td>2</td>
<td>All</td>
</tr>
<tr>
<td>11W nisin Z</td>
<td>3</td>
<td>47</td>
<td>All</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nisin A fragments (residues)</th>
<th>Induction capacity</th>
<th>Activity against M. flavus c</th>
<th>Rings present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin A</td>
<td>100</td>
<td>100</td>
<td>A–E</td>
</tr>
<tr>
<td>1–21</td>
<td>30</td>
<td>&lt;50</td>
<td>A–C</td>
</tr>
<tr>
<td>1–19</td>
<td>8</td>
<td>&lt;25</td>
<td>A–C</td>
</tr>
<tr>
<td>1–11</td>
<td>2</td>
<td>&lt;1</td>
<td>A, B</td>
</tr>
<tr>
<td>3–19 (L-Ala-5)</td>
<td>1</td>
<td>&lt;1</td>
<td>A–C</td>
</tr>
<tr>
<td>3–19 (o-Ala-5)</td>
<td>ND d</td>
<td>&lt;1</td>
<td>A–C</td>
</tr>
<tr>
<td>3–19</td>
<td>ND d</td>
<td>&lt;1</td>
<td>A–C</td>
</tr>
<tr>
<td>8–19</td>
<td>ND d</td>
<td>&lt;1</td>
<td>B, C</td>
</tr>
<tr>
<td>22–24</td>
<td>ND d</td>
<td>&lt;1</td>
<td>B, C</td>
</tr>
</tbody>
</table>

The induction capacity of nisin Z was taken as 100%; values were
calculated by measuring the distances between the dose-response
curves of nisin Z and each of the nisin species.

The minimal inhibitory concentration of nisin Z against L. lactis
MG1614 (14 ng/ml) or against M. flavus (11 ng/ml) was taken as 100%
activity. All nisin fragments contained the modified residues as they were
present in wild-type nisin A (Fig. 1B), unless indicated otherwise, at
position 5. Standard errors were <20% for each given value. A.U.,
arbitrary units.

Values are taken from Ref. 36.

ND, not detectable.

FIG. 2. Northern blot prepared using nisA as a probe of RNA
from several uninduced lactococcal cultures and cultures in-
duced with different amounts of nisin A or unmodified precur-
sor nisin A or with the lantibiotic Pep5. Lane 1, N29700 (nisin A
producer); lane 2, MG1614; lanes 3–7, N29800 with nisin A
(0, 1, 2.5, 10, and 50 ng/ml, respectively); lanes 8 and 9, N29800
ΔnisK with nisin A (0 and 2.5 ng/ml, respectively); lanes 10 and 11, N29700 ΔnisB
with nisin A (0 and 2.5 ng/ml, respectively); lane 12, N29800 with unmodi-
fied precursor nisin A (1000 ng/ml); lane 13, N29800 with Pep5
(1000 ng/ml).

Structural Requirements of the Inducer Molecule Tested by
Use of Synthetic Nisin Fragments—More detailed insight into

performing activity in membranes (40–42), while induction capacity
is likely to be dependent on interaction (directly or indi-
crectly) with NisK.

In further experiments, the nisin-producing strain N29700
with either plasmid pNZ2273 (containing the promoterless gusA
gene) or pNZ8008 (containing the nisA promoter fragment fol-
lowed by the gusA gene) was used in an agar diffusion assay (8)
to determine the amount of nisin produced. Fifty times lower
nisin production and severely reduced immunity were observed
when plasmid pNZ8008 was present compared with the situ-
uation where pNZ2273 was present. This can be explained by
titration of the response regulator NisR by the multicopy pres-
ence of the nisA promoter region containing the putative NisR-

binding site.
the minimal structural requirements of the inducer molecule was obtained by using synthetic nisin A fragments (35–39) in the gusA reporter assay (Table I). The minimal requirement for retaining induction capacity (2% induction of that of nisin A) was the presence of residues 1–11 of nisin A, comprising the first two rings. Addition of the third ring enhanced induction (8–30% induction), whereas a severe decrease in induction was caused by deleting the N-terminal residues Ile-1 and dehydrobutyrine 2 (0–1% induction) (Table I). Fragments that contained rings B and C or rings D and E (for nomenclature of rings, see Fig. 1B) were not capable of acting as a signal effector. Thus, the most probable site of molecular interaction with the sensor protein NisK will be residues 1–11 of the nisin molecule.

Requirement of nisK Expression for Signal Transduction—The sequence of the nisK gene located on Tn5276 has been reported (43) and was found to be identical to that of nisK from L. lactis 6F3 (10). The chromosomal nisK gene was insertionally inactivated by introduction of an erythromycin resistance gene (28) into strain NZ9800, yielding strain NZ9805. As expected, transcription of ΔnisA was no longer inducible by any of the nisin species (Fig. 2, lanes 8 and 9). Nisin production in strain NZ9805 could not be restored by introduction of plasmid pNZ9010 (nisA) or pNZ9013 (nisZ), whereas it could be restored in strain NZ9800. Since the immunity level of strain NZ9805 is similar to that of strain MG1614 (0.01 μg of nisin A/ml), induction experiments were performed with amounts of nisin well below this level. Under these conditions, normal growth of the cells was observed. Strain NZ9805 was also transformed with pNZ28008, but after induction with 0.0005–0.0025 μg of nisin A/ml, no β-glucuronidase activity could be measured (<0.3 arbitrary unit), indicating at least 200 times lower expression than in strain NZ9800 containing pNZ28008, with the same inducer concentrations (Fig. 3). No polar effects of the nisK disruption on expression of the nisFEG genes downstream of nisK are expected since a promoter has been indicated in front of nisFEG (11). Moreover, the nisR and nisK genes have been integrated on the chromosome of strain MG1614 by replacement recombination, and the resulting strain was transformed by pNZ28008. In this strain, gusA expression was inducible by nisin species (data not shown), proving that only nisR and nisK are required for signal transduction. These results clearly demonstrate that NisK is essential in the signal transduction pathway and probably interacts directly with nisin itself.

Effects of Disruption of nisB on Transcription of nisA and Downstream Genes—An in-frame deletion in the nisB gene of L. lactis strain NZ9700, made by replacement recombination, abolished nisin production as well as transcription of nisA, demonstrating that a hampered biosynthesis of nisin abolishes transcription of nisA. In this case, transcription of nisA could be restored by addition of nisin to the cells (Fig. 2, lane 11), probably because of the presence of intact nisR and nisK genes, which have their own promoter. The transcription start site of nisRK was mapped by primer extension and shown to be an A nucleotide 26 nucleotides upstream of the start codon of nisR (position 2117 in the nucleotide sequence published in Ref. 8). To probe the influence of a large inverted repeat sequence located in the intergenic region between ΔnisA and nisB on expression of genes downstream of ΔnisA (Fig. 1A), another plasmid was constructed (pNZ28002) in which the nisin promoter fragment including AnisA as well as the intergenic region and the first part of nisB was fused to the gusA gene. This plasmid was able to direct expression of gusA in strain NZ9800 only after induction with nisin species, albeit to an ∼50-fold reduced level relative to gusA expression in pNZ28008 in strain NZ9800. When the nisin promoter fragment was removed from pNZ28002, yielding pNZΔ8002, β-glucuronidase activity was completely abolished, even in the presence of an inducer. These results show that expression of at least one downstream gene, i.e. nisB, is coregulated and is dependent on the presence of the nisA promoter. Most likely, expression of the other downstream genes nisBTCIP limited read-through is also dependent on the nisA promoter since a significant increase in immunity levels, for which NisI is partially responsible (9), was found in the induced state relative to the uninduced state of strain NZ9800. Moreover, no apparent promoter sequences were found in front of any of the genes nisBTCIP.

Conclusion—We have demonstrated that transcription of nisA is autoregulated, not intracellularly by its direct translation product, but extracellularly by the secreted and fully modified peptide via signal transduction by a two-component regulatory system. A model based on previous work (5–11) and on this study shows the possible sequence of events with regard to nisin biosynthesis and regulation (Fig. 4).

Mutants of nisin or precursors of nisin that have the leader peptide attached to the mature lantibiotic (second molecule shown in Fig. 1B) can also act as inducers, whereas other antimicrobial peptides are incapable of induction. The presence of the modified residues is of crucial importance for induction capacity, especially those present in the N-terminal part of nisin. To our knowledge, this is the only report of peptides that can induce transcription of their own structural gene via signal transduction. Interestingly, a recent report on syndecan biosynthesis in mice, which plays a role in wound repair, describes the role of the antimicrobial peptide PR39 in induction of syndecan gene transcription (44), although the amount of inducer needed (0.5 μM) is at least a factor of 10,000 higher than for nisin (30 pm). This suggests that the role of antimicrobial peptides in nature might be broader than just the antagonistic action because in some cases these peptides can also act as signals for transcription activation of their own structural gene.
or of other genes. There may be several evolutionary reasons for the autoregulation of nisin gene transcription via signal transduction, e.g. (i) to save energy by control of the integrity of the gene cluster since any dysfunctional biosynthetic gene will abolish inducer formation and thus expression of biosynthetic genes; (ii) to raise immunity levels in response to high nisin production by neighboring cells, in other words, to amplify the response to environmental signals; or (iii) to promote cell to cell communication that allows the production of antimicrobial peptides in high quantities in a concerted action, thereby decreasing the chance of resistance development in target organisms.

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